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INCORPORATION OF [^{3}H]PALMITATE INTO DISATURATED PHOSPHATIDYLCHOLINES IN ALVEOLAR TYPE II CELLS ISOLATED BY CENTRIFUGAL ELUTRIATION

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In order to study synthesis of pulmonary surfactant materials, we measured incorporation of [^{3}H]palmitate into disaturated phosphatidylcholines (PC) in alveolar type II cells isolated by centrifugal elutriation. The time course for this process is not linear and, at high external palmitate levels (1 mM), incorporation is maximal in 4–5 h. Incorporation is dependent on extracellular palmitate with a V_{max} (at 1 mM) of 1.66 nmol palmitate incorporated into disaturated PC/$4.2 \cdot 10^5$ cells per 2 h and a $K_{1/2}$ of 0.1 mM palmitate. Addition of an optimal amount of extracellular choline (0.05 mM) increases V_{max} and decreases $K_{1/2}$ for palmitate. Incorporation of palmitate is dependent upon cell number, inhibited by extracellular Ca^{2+} and stimulated by external Mg^{2+}. Cholinergic and β-adrenergic agonists do not increase incorporation. Pulmonary lavage fluid inhibits incorporation of palmitate into disaturated PC, suggesting there is negative feedback involved. Disaturated PC which has been recently synthesized (i.e., over a 2 h period) is broken down intracellularly by type II cells when they are suspended in palmitate-free medium. These results indicate that (1) several factors, such as substrate levels, cell number, Ca^{2+}, Mg^{2+} and amount of surfactant present, are involved in the regulation of palmitate incorporation into disaturated PC; (2) disaturated PC which has been recently synthesized may be broken down by type II cells; and (3) surfactant synthesis in freshly isolated cells differs slightly from that reported by other investigators in type II cells maintained in primary cell culture.

Introduction

Pulmonary surface-active materials are a mixture of lipids, proteins and carbohydrates which lines the alveoli and prevents their collapse by lowering surface tension forces. It has been established that the synthesis of these materials occurs primarily in alveolar type II cells [1–5]. Recently, techniques have been developed to isolate type II cells from other types of pneumocytes and both synthesis [1–3] and release [4,5] of pulmonary surfactant have been investigated in these isolated cell preparations. In these studies, type II cells were isolated from other types of lung cells by various techniques, all of which included maintaining the cells in primary cell culture for approx. 24 h. As far as we know, no studies of surfactant synthesis have been done in freshly isolated alveolar type II cells.

The component of pulmonary surfactant which is present in the greatest amount and which is the major surface-active material is DPPC [6,7]. DPPC is produced in alveolar type II cells from substrates such as choline, glucose, glycerol, acetate and palmitate [1–3,8,9]. In fact, synthesis of surfactant has been studied by measuring the in-

Abbreviations: PC, phosphatidylcholine(s); DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol.

corporation of one or more of these substrates into DPPC [1–3,8,9]. With such studies, some of the factors which may be involved in regulating synthesis have been identified. For example, synthesis is dependent on the extracellular concentration of palmitate [1–3,8], β-adrenergic agonists increase synthesis [10] and release [4,5,11,12] of surfactant, and the hormones, dexamethasone and hydrocortisone, increase the synthesis of pulmonary surfactant materials [13–15]. Although these observations have increased our understanding, the mechanisms involved in the regulation of pulmonary surfactant synthesis still remain largely unknown.

In this paper we report the results of experiments in which pulmonary surfactant synthesis was studied in freshly isolated alveolar type II cells, i.e., cells which have not been maintained in primary cell culture. It is possible that synthesis may be somewhat different in these cells, especially since there are noticeable changes in the ultrastructure [16] and the ability of the cells to synthesize phosphatidylglycerol [3] following the cell culture process. In these experiments we measured the incorporation of [^{3}H]palmitate into disaturated PC in type II cells isolated via centrifugal elutriation. The objectives of this investigation were (1) to study some factors which may be involved in the regulation of pulmonary surfactant synthesis, e.g., substrate availability, enzyme levels, level of product, calcium and magnesium, and chemical mediators; and (2), whenever possible, to compare our results with those obtained by other investigators who used cells isolated by primary cell culture. A preliminary report of these findings has appeared previously [17].

Methods

Preparation of isolated alveolar type II cells

Type II cells were isolated by elastase digestion and purified by centrifugal elutriation as described previously [18]. Briefly, male Sprague-Dawley rats (200–300 g) were anesthetized with sodium pentobarbital (65 mg/kg body weight) and the heart and lungs were removed en bloc. Lungs were perfused with 0.9% NaCl to remove blood cells. Free alveolar macrophages were removed by tracheal lavage with phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4 and 5.5 mM glucose, pH 7.4) according to the method of Myrvik et al. [19]. Lungs were then filled with elastase solution (40 U/ml type I elastase and 0.006% DNAase (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered medium) and incubated at 37°C for 30 min to free lung cells [20]. Following enzymatic digestion, lungs were minced with a tissue chopper (slice thickness 0.5 mm) and digestion was arrested by incubation at 37°C for 10 min in 20 ml phosphate-buffered medium containing 25% fetal calf serum and 0.006% DNAase. Type II cells were then purified via centrifugal elutriation [21,22]. The cell suspension was strained through nylon mesh and loaded into an elutriation rotor (Beckman Model J-21 centrifuge equipped with a Model JE-6 rotor; Beckman Instrument Co, Fullerton, CA) [23] at a flow rate of 10 ml/min and a rotor speed of 2000 rpm. Then 200 ml phosphate-buffered medium containing 0.5% bovine serum albumin were infused through the elutriator at this flow rate and rotor speed to remove cell fragments and small pneumocytes. Bovine serum albumin was used to prevent the cells from clumping [24]. Type II cells were then recovered at a flow rate of 18.9 ml/min and a rotor speed of 2000 rpm. The type II cell-enriched fraction was harvested by centrifugation at $1000 \times g$ for 5 min and resuspended in phosphate-buffered medium containing 0.006% DNAase.

Cell number was determined with a Coulter Model ZB electronic cell counter (Coulter Instrument Co., Hialeah, FL) and mean cell volume was measured with a channelizer attachment for the Coulter Counter. The viability of the cells was estimated by measuring oxygen consumption with a Gilson K-IC oxygraph fitted with a Clark electrode. The oxygraph was calibrated by bubbling gases of known oxygen content through the phosphate-buffered medium until saturation occurred and then measuring the oxygen level. Oxygen consumption was measured in a suspension containing $1 \cdot 10^6$ cells in 1.65 ml phosphate-buffered medium with DNAase and expressed as nmol oxygen consumed/10^6 cells per h. Oxygen consumption in our cell preparations was 215 ($\pm$40) nmol/10^6 cells per h (mean value $\pm$ S.E. for 10 experiments) and this value was not increased by

the addition of 5 mM sodium succinate. These results indicate that the type II cells used in these experiments were viable and that the cell membranes were intact.

The purity of the type II cell-enriched fraction was routinely estimated with the fluorescent dye, phosphine 3R, as we have reported previously [18]. Mason et al. [24] have shown that this dye is concentrated by the lamellar bodies of type II cells. In our experiments, 9 parts of a cell suspension were added to 1 part of 0.02% phosphine 3R (Roboz Surgical Instrument Co., Washington, DC) solution. The dye was allowed to equilibrate with the cells for 2 min. Then 40 μl of this suspension were viewed under a Zeiss universal fluorescence microscope (Carl Zeiss Co., F.R.G.) set at an activation wavelength of 477 nm and an emission wavelength of 512 nm. Both type II cells and the total number of cells were counted. In each experiment, enough fields were counted to bring the total number of cells counted to 300. The results were also verified with electron and light microscopy. In the experiments reported in this paper we obtained 1.9 $(\pm 0.2) \cdot 10^7$ cells per rat in the type II cell-enriched fraction with a purity of 85 ($\pm$ 1)% (mean values $\pm$ S.E for 30 experiments). The 10–15% contamination was due to polymorphonuclear leukocytes (5–10%) and alveolar macrophages (5%).

Incubation of cells and preparation of samples for analysis

Incorporation of [^{3}H]palmitate into disaturated PC was determined by incubating type II cells in medium containing various concentrations of palmitic acid with trace amounts of [^{3}H]palmitate and measuring the amount of radioactivity in the disaturated PC which was produced. After isolation of the type II cells as described above, the cells were suspended in phosphate-buffered medium containing DNAase and an appropriate amount of palmitate. The palmitic acid (Sigma) was complexed with bovine serum albumin in a molar ratio of 5.3 : 1 (fatty acid/bovine serum albumin) as described by Hendry and Possmayer [25]. In most experiments the number of cells used was $8.3 \cdot 10^5$ cells per ml. At the start of the experiment, 10^{-4} μmol [9,10-^{3}H]palmitic acid (spec. act. 11.8 Ci/mmol; New England Nuclear Corp., Boston, MA) was added to each sample and the cell suspensions were incubated at 37°C for varying lengths of time. Following the incubation period, a 0.5 ml aliquot of the cell suspension was mixed well with 10 ml chloroform/methanol (2 : 1, v/v). 1 mg of mixed lipids isolated from rat lungs was added to each sample of cell suspension and these samples were saved for analysis. In one series of experiments incorporation of choline into disaturated PC was measured by adding 1 μCi [*methyl*-^{3}H]choline chloride (spec. act. 80 Ci/mmol; New England Nuclear) to the incubation medium.

In some experiments we determined the effects of lipid vesicles on incorporation of [^{3}H]palmitate into disaturated PC. The lipids employed and the concentrations in the incubation medium were: 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC, 0.233 mg/ml); cholesterol (0.039 mg/ml); 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (16 : 0/18 : 1, 0.050 mg/ml); 1-oleoyl-2-palmitoyl-*sn*-phosphatidylcholine (18 : 1/16 : 0, 0.030 mg/ml; Sigma); 1-palmitoyl-2-palmitoleoyl-*sn*-phosphatidylcholine (16 : 0/16 : 1; 0.029 mg/ml); 1-stearoyl-2-oleoyl-*sn*-phosphatidylcholine (18 : 0/18 : 1; 0.004 mg/ml; Applied Science, State College, PA); and phosphatidylglycerol (DPPG; 0.030 mg/ml; Supelco, Inc., Bellefonte, PA). The lipids were added to the incubation medium as described previously [26,27]. Briefly, the lipids were dissolved in 50 μl of ethanol. Liposomes were formed by the rapid injection of the dissolved lipids with a Hamilton syringe into incubation medium warmed to 48°C. Then the dispersion was sonicated for 30 s with a probe sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) set at the microtip limit.

In one series of experiments we studied the effects of chemical mediators on disaturated PC synthesis. The cells were incubated in phosphate-buffered medium (containing 0.1 mM palmitate, 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$) containing a final concentration of 10^{-4} M drug. The following drugs were obtained from Sigma Chemical Co., L-epinephrine bitartrate, L-isoproterenol D-bitartrate, N^6,$O^{2'}$-dibutyryladenosine 3' : 5'-cyclic monophosphate, N^2,$O^{2'}$-dibutyrylguanosine 3' : 5'-cyclic monophosphoric acid, acetyl-β-methylcholine chloride, acetylcholine bromide, atropine sulfate and physostigmine. Terbutaline sulfate was

obtained from Astra Pharmaceutical Products, Inc. (Worcester, MA).

Isolation of disaturated PC and determination of radioactivity

Disaturated PC were isolated from the samples, which were prepared as described above, according to the method of Mason et al. [28]. Briefly, total lipids were extracted with chloroform/methanol (2 : 1, v/v), the solvent was evaporated, and the lipids were reacted with osmium tetroxide dissolved in CCl_4. Following evaporation of the CCl_4, the samples were dissolved in chloroform/methanol (20 : 1, v/v) and placed on a column of neutral alumina (100–200 mesh, BioRad Laboratories, Richmond, CA). The disaturated PC was eluted from the columns with chloroform/methanol/7 M ammonium hydroxide (70 : 30 : 2, v/v). After evaporation of the solvent, 10 ml of Aquasol (New England Nuclear) were added to each sample of disaturated PC and the samples were counted in the tritium channel of a liquid scintillation spectrometer (Model 3380, Packard Instrument Co., Downers Grove, IL). With this isolation procedure we were able to obtain greater than 98% recovery of [^{14}C]DPPC. Furthermore, we established that the amount of [^{3}H]palmitate recovered in the disaturated PC fraction which was not incorporated into disaturated PC was less than 0.5% of that added initially. The results of our experiments were expressed as nmol palmitate or choline incorporated into disaturated PC.

Phosphorus determinations

The amount of phosphorus in lipids extracted from various samples of pulmonary lavage fluid and isolated type II cells was determined by the method of Bartlett [29]. All steps were carried out using disposable glassware. Briefly, following extraction of the lipids with chloroform/methanol (2 : 1, v/v), each sample was evaporated to dryness and 3.3 N H_2SO_4 (1.5 ml) was added. The samples were heated at 150–160°C for 3 h, 30% H_2O_2 (three drops) was added, and then the samples were maintained at 150°C for an additional 90 min. Ammonium molybdate (4.6 ml of a 0.22% solution) and Fiske-SubbaRow reagent (0.2 ml) were added to each sample and they were heated at 100°C for 7 min. Phosphorus was determined by reading the absorbances at 830 nm against standards. In order to obtain phospholipid content, lipid phosphorus values were multiplied by 25 [12].

Results

Time course for incorporation

Time courses for incorporation of [^{3}H]palmitate into disaturated PC were determined at various external concentrations of palmitate and over different periods of time. The results are shown in Fig. 1. Incorporation becomes maximal more rapidly at lower palmitate concentrations than at higher levels; e.g., at 0.001 mM palmitate incorporation is maximal after only 1 h of incubation while at 1.0 mM substrate a time period of 5 h is required for maximal incorporation. None of the time courses is linear. The nonlinear time courses are not due to deterioration of the cells, since measurements of oxygen consumption and membrane integrity indicate that the cells remain viable for at least 7 h under these incubation conditions. These results obtained in freshly isolated cells

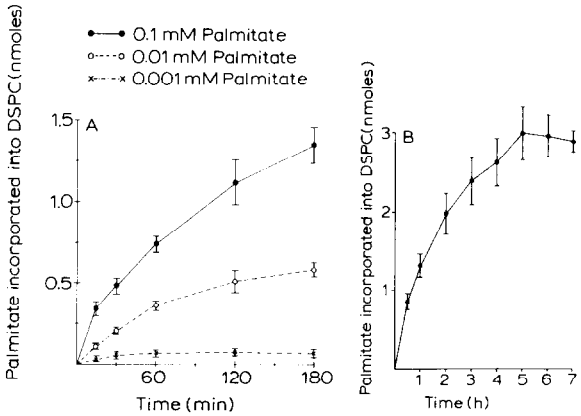

Fig. 1. Time courses for incorporation of [^{3}H]palmitate into disaturated PC in isolated alveolar type II cells suspended in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ and different concentrations of palmitate. Incorporation was measured in samples (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells. Measurements made over a 3 h incubation period at 0.001 (×–·–·–×), 0.01 (O- - - - -O) and 0.1 mM (●———●) palmitate are shown in panel A. Incorporation measured over a 7 h period at 1.0 mM external palmitate is shown in panel B. The points are mean values for three to six experiments and the bars represent the standard errors of the means.

differ from those obtained in type II cells which had been maintained in primary culture [1] and in cells maintained in organotypic culture [30,31], in which palmitate incorporation was linear over a 3 h incubation period.

Dependence of incorporation on substrate levels and cell number

The dependence of incorporation of [^{3}H]-palmitate into disaturated PC on the extracellular palmitate concentration is shown in Fig. 2. The measurements were made following an incubation period of 2 h. The relationship between external palmitate and incorporation displays saturation kinetics. Maximal incorporation and the $K_{1/2}$ value (the extracellular palmitate level at which one-half maximal incorporation occurs), obtained from a double-reciprocal plot, are 1.66 nmol palmitate incorporated into disaturated PC and 0.10 mM, respectively (Table I). These results also differ from those obtained with cells maintained in culture. Maximal incorporation occurs at 0.1 mM palmitate in cells isolated by primary culture [1] and at 15 μM palmitate in cells maintained in organotypic culture [31]. In our experiments, maximal incorporation occurs at 0.5–1.0 mM palmitate. Furthermore, the amount of [^{3}H]palmitate incorporated into disaturated PC is 2–3-times greater in cells isolated via primary cell culture [1]. In most of the remaining experiments in this paper we used an extracellular palmitate level of 0.1 mM, a concentration which approximates the $K_{1/2}$ value.

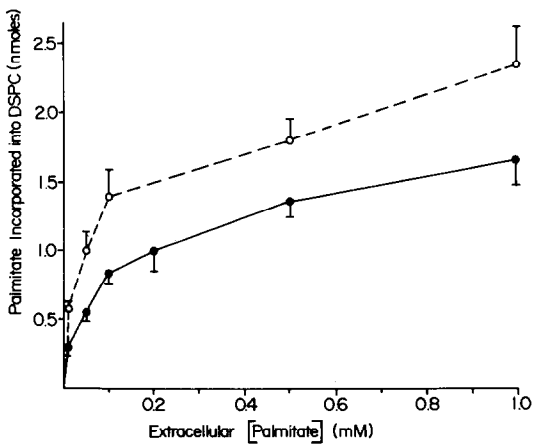

Fig. 2. Incorporation of [^{3}H]palmitate into disaturated PC as a function of the extracellular palmitate concentration in the absence (●———●) and presence (○------○) of choline (0.05 mM). The incubation medium consisted of phosphate-buffered medium with 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, varying amounts of palmitate and choline (where indicated). The measurements were made from samples of cell suspension (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells after an incubation period of 2 h. The points are mean values for six experiments and the bars represent the standard errors of the means.

TABLE I

EFFECTS OF CHOLINE ON INCORPORATION OF [^{3}H]PALMITATE INTO DISATURATED PC

These values were obtained from double-reciprocal plots using the data shown in Fig. 2. The data were fitted with straight lines by linear regression analysis to obtain V_{max} (i.e., the maximum rate of incorporation) and $K_{1/2}$ (the concentration of external palmitate at which one-half maximal incorporation occurs). R is the correlation coefficient for these lines.

Substrate	V_{max} (nmol palmitate incorporated into disaturated PC	$K_{1/2}$	R
Palmitate alone	1.66	0.10	0.9931
Palmitate + choline (0.05 mM)	2.24	0.06	0.9827

The effect of choline, another substrate for disaturated PC synthesis, on incorporation at different levels of external palmitate is also shown in Fig. 2. The maximal effect of choline is obtained at a concentration of 0.05 mM, the level which was used in these experiments. Note that in the presence of choline incorporation of palmitate into disaturated PC is increased at all levels of extracellular palmitate. In fact, the effects of choline on the palmitate-concentration dependence of incorporation are to increase maximal incorporation and to decrease the $K_{1/2}$ value for extracellular palmitate (Table I). Thus, in the presence of choline incorporation of palmitate into disaturated PC is enhanced.

The effect of cell number on surfactant synthesis was studied by measuring the incorporation of palmitate into disaturated PC while using different numbers of type II cells. In these experiments the level of palmitate in the medium was 0.1 mM. The results indicate that a linear relationship exists between incorporation and cell number over a range of $1 \cdot 10^5$–$2.5 \cdot 10^6$ cells per sample. The correlation coefficient for the line drawn from linear regression analysis is 0.9974. In most of the experiments reported in this paper we used $4.2 \cdot 10^5$

cells per sample, a number which is well within the linear range. Since the enzyme levels vary with cell number, these results indicate that there is a linear relationship between the level of enzymes involved in surfactant synthesis and incorporation of palmitate in disaturated PC.

Dependence on calcium and magnesium

The effects of extracellular calcium and magnesium on the incorporation of palmitate into disaturated PC are shown in Table II. These experiments were performed at an external palmitate level of 0.1 mM. Addition of Ca^{2+} (1.8 mM) alone to the incubation medium inhibits incorporation. On the other hand, addition of Mg^{2+} (1.0 mM) alone has a small stimulatory effect. When both Ca^{2+} and Mg^{2+} are included in the incubation medium, incorporation is no different from when both divalent ions are absent. Thus, Ca^{2+} alone inhibits incorporation of palmitate into disaturated PC. The inhibition is abolished by the addition of Mg^{2+}. The reasons for these effects are not yet known.

Effects of pulmonary lavage fluid

In order to determine the effects of the products of surfactant synthesis on the incorporation of palmitate into disaturated PC, pulmonary lavage fluid was added to the incubation medium. Lavage fluid was obtained by repeated injections of the same 3 ml of phosphate-buffered medium into the right and left lungs. After removal of alveolar macrophages by centrifugation at 500 $\times g$ for 5 min, lavage fluid was added to the incubation medium which contained 0.1 mM palmitate, 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$. The amount of lavage fluid added is expressed in mg phospholipid. In a separate set of experiments we determined that the lavage fluid has an osmolarity of 310 mosM, a pH of 7.4, and has no effect on the oxygen consumption of cells incubated in palmitate-free medium. Thus, this material does not appear to harm the cells.

A dose-response curve for the effects of pulmonary lavage fluid is shown in Fig. 3. Lavage materials produce a dose-dependent inhibition of incorporation of palmitate into disaturated PC. In the most concentrated form we could obtain, about 0.45 mg phospholipid per ml, there is greater than 80% inhibition. We also determined that this inhibition can be reversed by removing the lavage materials and resuspending the cells in fresh incubation medium (data not shown). Thus, these results suggest that some component(s) of pulmonary surfactant materials inhibits incorporation of palmitate into disaturated PC.

It is possible that apparent inhibition of incorporation of [^{3}H]palmitate into disaturated PC by lavage materials is due to the presence of free palmitate in the lavage fluid, i.e., dilution of the specific activity of palmitate. In order to test this possibility, we determined the effect of lavage materials on the incorporation of [^{3}H]choline into disaturated PC. The results are shown in Table III. Lavage fluid produces about 75% inhibition of choline incorporation, i.e., about the same effect as with incorporation of palmitate. An almost identical amount of inhibition is produced by the lipid extract of lavage fluid. The extraction procedure results in the elimination of 98 (± 2)% of the free choline. Therefore, these results indicate that the presence of pulmonary surfactant materials inhibits synthesis of disaturated PC and that the inhibition is not due to dilution of specific activity of palmitate or choline. Furthermore, the material(s) which produces the inhibition is extracted with the lipid components.

The effects of some of the individual lipid com-

TABLE II

EFFECTS OF CALCIUM AND MAGNESIUM ON THE INCORPORATION OF [^{3}H]PALMITATE INTO DISATURATED PC

Experiments were carried out in phosphate-buffered medium containing 0.1 mM palmitate and the amounts of $CaCl_2$ and $MgCl_2$ shown in the table. The measurements were made from samples of cell suspension (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells after an incubation period of 2 h. The amount of palmitate incorporated into disaturated PC in the absence of Ca^{2+} and Mg^{2+} was 1.10 (± 0.08) nmol. The values are means for five experiments $\pm$ S.E.

Extracellular [Ca^{2+}] (mM)	Extracellular [Mg^{2+}] (mM)	Incorporation of [^{3}H]palmitate into disaturated PC (% control)
0	0	100
1.8	0	62 $\pm$ 3
0	1.0	120 $\pm$ 7
1.8	1.0	106 $\pm$ 10

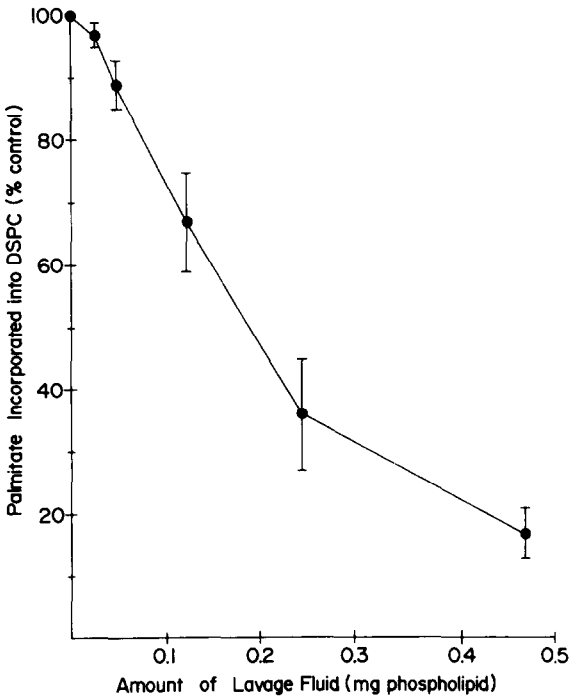

Fig. 3. Effects of materials obtained from pulmonary lavage on incorporation of palmitate into disaturated PC. Lavage fluid was obtained by repeated injections and withdrawals of the same 3 ml of phosphate-buffered medium into the right and left lungs. Alveolar macrophages were removed from the fluid by centrifugation. The incubation medium for these experiments contained phosphate-buffered medium with 0.1 mM palmitate, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ and varying amounts of lavage fluid (expressed as mg phospholipid). Incorporation was measured in samples of cell suspension (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells after incubation for 2 h. The control value (i.e., no lavage fluid present) is 0.91 ($\pm$0.21) nmol palmitate incorporated into disaturated PC. The points are mean values for five experiments and the bars represent the standard errors of the means.

ponents of pulmonary surfactant materials on the incorporation of palmitate into disaturated PC were determined and the results are shown in Table IV. The substances tested include DPPC, four unsaturated phosphatidylcholines (16 : 0/18 : 1, 18 : 1/16 : 0, 16 : 0/16 : 1 and 18 : 0/18 : 1), DPPG and cholesterol. The amounts of the lipids added to the incubation medium were calculated to be approximately the same as those present in 1 ml of the lavage fluid we obtained [9,32]. All of the substances tested seem to produce some inhibition of incorporation, although the DPPC effect is not

TABLE III

EFFECTS OF PULMONARY LAVAGE FLUID ON INCORPORATION OF [^{3}H]CHOLINE INTO DISATURATED PC

Experiments were performed in phosphate-buffered medium containing 0.1 mM palmitate, 0.05 mM choline, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ and the amounts of lavage fluid or lipid extract of lavage fluid (expressed as mg phospholipid) shown. [^{3}H]Choline (1 μCi [*methyl*-^{3}H]choline chloride; spec. act. 80 Ci per mmol; New England Nuclear) was added to samples of cell suspension containing $8.4 \cdot 10^5$ cells per ml. After incubation for 4 h at 37°C, measurements were made from samples of cell suspension (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells. The amount of choline incorporated into disaturated PC in control cells was 2.08 ($\pm$0.39) nmol. The values are means for six experiments $\pm$ the S.E.

Treatment	mg phospholipid in incubation medium	Incorporation of [^{3}H]choline into disaturated PC (% control)
Control	–	100
Lavage fluid	0.46 $\pm$ 0.02	25($\pm$5)
Lipids from lavage fluid	0.46 $\pm$ 0.03	18($\pm$3)

TABLE IV

EFFECTS OF SOME COMPONENTS OF PULMONARY LAVAGE FLUID ON INCORPORATION OF [^{3}H]-PALMITATE INTO DISATURATED PC

Experiments were performed in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.1 mM palmitate. The lipids were dissolved in 0.05 ml ethanol, injected into the medium (at 48°C) with a Hamilton syringe and sonicated for 30 s. The measurements were made from samples of cell suspension (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells after an incubation period of 2 h at 37°C. The amount of palmitate incorporated into disaturated PC in control cells was 1.90 ($\pm$0.16) nmol. The values are means for six experiments $\pm$ the S.E. The unsaturated phosphatidylcholines (mg/ml) used were 16 : 0/18 : 1 (0.050), 18 : 1/16 : 0 (0.030), 16 : 0/16 : 1 (0.029) and 18 : 0/18 : 1 (0.004).

Component	Amount (mg/ml)	Incorporation of [^{3}H]palmitate into disaturated PC (% control)
Control	–	100
DPPC	0.233	87 ($\pm$11)
Unsaturated PC	0.113	70 ($\pm$7)
DPPG	0.030	70 (+4)
Cholesterol	0.039	80 ($\pm$9)
All components	0.415	71 ($\pm$5)

significant. DPPG and the unsaturated phosphatidylcholines produce the most inhibition, i.e., about 30%. Even if all lipid components are included, the inhibition which results is still only about 30%. These results indicate that some, but not all, of the inhibition of incorporation produced by pulmonary lavage fluid can be duplicated by using vesicles of the lipid components of surfactant materials.

Effects of chemical mediators

Other investigators have shown that release of pulmonary surfactant materials is enhanced by β-adrenergic and cholinergic stimulation in whole lung [11,12] and in isolated alveolar type II cells [4,5]. Therefore, we studied the effects of some of these substances on the incorporation of palmitate into disaturated PC in type II cells. The results are shown in Table V. None of the substances tested has any significant effect on surfactant synthesis, with the exception of acetylcholine. However, the acetylcholine effect is blocked by atropine, an inhibitor of acetylcholine binding, and by physostigmine, an acetylcholinesterase inhibitor. In addition, another cholinergic agonist, methacholine, which is not broken down by acetylcholinesterase, has no effect. Thus, the acetylcholine effect seems to be due to stimulation by choline, a breakdown product of acetylcholine when it is metabolized by acetylcholinesterase. It also appears that acetylcholine must be bound to the type II cell receptors in order for the acetylcholinesterase to work, since atropine inhibits the acetylcholine effect. These results suggest that there is no direct stimulation of disaturated PC synthesis in type II cells by β-adrenergic or cholinergic agonists.

TABLE V

EFFECTS OF CHEMICAL MEDIATORS ON INCORPORATION OF [^{3}H]PALMITATE INTO DISATURATED PC

Experiments were performed in phosphate-buffered medium containing 0.1 mM palmitate, 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$. The concentration of chemical mediator used was 10^{-4} M. Measurements were made from samples of cell suspension (0.5 ml total volume) which contained $4.2 \cdot 10^5$ cells after an incubation period of 4 h. The amount of palmitate incorporated into disaturated PC in control cells was 1.24 (±0.11) nmol. The values are means for five experiments ± the S.E.

Treatment	Incorporation of [^{3}H]palmitate into disaturated PC (% control)
Control	100
Epinephrine	98 ± 4
Isoproterenol	92 ± 3
Terbutaline	101 ± 8
Dibutyryl-cyclic AMP	97 ± 3
Cyclic GMP	98 ± 12
Methacholine	89 ± 6
Acetylcholine	140 ± 4
Acetylcholine + atropine	110 ± 3
Acetylcholine + physostigmine	104 ± 5

Fate of recently synthesized disaturated PC

In this paper we have shown that at high external levels of palmitate (1 mM) incorporation is not maximal for 4–5 h. The following experiments were performed in order to determine the fate of [^{3}H]palmitate-labeled disaturated PC after only 2 h of synthesis in vitro. Type II cells were incubated with [^{3}H]palmitate for 2 h, centrifuged, washed free of palmitate, and resuspended in palmitate-free medium. The amount of radioactivity present in disaturated PC was measured in the intra- and extracellular compartments over an additional 3 h period. The labeled disaturated PC in the medium is only 2–5% of that found in the cells and does not change during the incubation period. The intracellular labeled disaturated PC diminishes with time such that only 40–70% of the

TABLE VI

EFFECTS OF TEMPERATURE AND PALMITATE ON RECENTLY SYNTHESIZED INTRACELLULAR DISATURATED PC

Type II cells were incubated in phosphate-buffered medium with [^{3}H]palmitate for 2 h, centrifuged, washed free of palmitate and resuspended in each of the media shown below. The intracellular labeled disaturated PC was measured after a 3 h incubation period. The results are expressed as a percent of the intracellular labeled disaturated PC which was present before the 3 h incubation. The values shown are means for five experiments (± S.E.).

Treatment	Intracellular labeled disaturated PC after 3 h incubation (% zero time)
Palmitate-free medium (37°C)	41 (±2)
Palmitate-free medium (2°C)	91 (±2)
1 mM palmitate (37°C)	71 (±12)

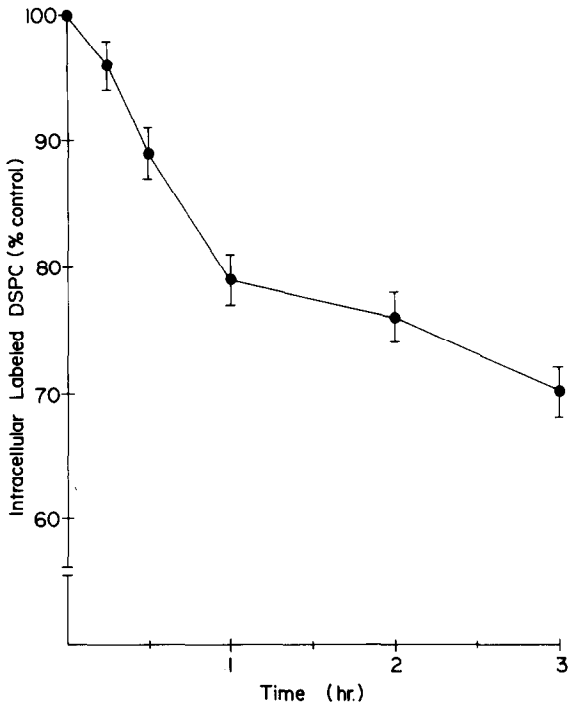

Fig. 4. Time course for the disappearance of recently synthesized intracellular disaturated PC. Type II cells were incubated in phosphate-buffered medium containing 1.8 mM Ca^{2+}, 1.0 mM Mg^{2+} and [^{3}H]palmitate (total palmitate = 1 mM) for 2 h. The cells were then spun down, washed three times, and resuspended in fresh palmitate-free phosphate-buffered medium (with Ca^{2+} and Mg^{2+}). The amounts of labeled disaturated PC in the intra- and extracellular compartments were measured over a 3 h incubation period at 37°C. The labeled disaturated PC in the medium was small (2–5% of intracellular disaturated PC) and did not change with time. The intracellular disaturated PC shown above is expressed as a percent of that present before the start of the 3 h incubation in the absence of external palmitate. The measurements were made from samples containing $4.2 \cdot 10^5$ cells. The points are mean values for six experiments and the bars represent the standard errors of the means.

initial amount is present after 3 h of incubation in palmitate-free medium (Fig. 4 and Table VI). The disappearance of disaturated PC is prevented if the cells are maintained at 2°C and the rate of disappearance is slowed if palmitate (unlabeled) is included in the medium (Table VI). In a separate set of experiments (data not reported) we determined that when labeled choline is incorporated into disaturated PC, the intracellular labeled disaturated PC becomes diminished to the same extent; i.e., the palmitate/choline ratio remains the same during the disappearance of disaturated PC. Thus, these data do not reflect simple exchange of label; the intracellular disaturated PC is actually metabolized. These results suggest that intracellular disaturated PC which has been recently synthesized can be broken down by type II cells.

Discussion

The results of these experiments provide information about synthesis of pulmonary surfactant materials in freshly isolated alveolar type II cells, i.e., cells which have not been maintained for extended periods of time in primary cell culture. We measured the incorporation of [^{3}H]palmitate into disaturated PC. These results provide: (1) a basis for comparison with results obtained by other investigators who used cells isolated with tissue culture techniques and (2) some new information about some factors which may be involved in the regulation of pulmonary surfactant synthesis, e.g., substrate availability, enzyme levels, extracellular calcium and magnesium, and level of product.

Our results indicate that there are some differences between freshly isolated type II cells and cells isolated via cell culture techniques. For example, the time course and magnitude of the incorporation of palmitate into disaturated PC and the dependence of incorporation on extracellular palmitate are different. In freshly isolated type II cells the time course for incorporation is not linear, while in cells maintained in primary culture [1] and in organotypic culture [30,31] incorporation is linear for 3–4 h. Batenburg et al. [1] reported that the maximal rate of incorporation in primary culture is about 5 nmol palmitate per $5 \cdot 10^5$ type II cells. In freshly isolated cells we obtained only about 2 nmol palmitate incorporated into disaturated PC per $5 \cdot 10^5$ cells, indicating that the magnitude of incorporation is 2–3-times greater in cultured cells. The relationship between incorporation and the extracellular palmitate concentration displays saturation kinetics in all type II cell preparations. However, there are some quantitative differences. Maximal incorporation occurs at 0.5 mM palmitate in freshly isolated cells and at 0.1 mM and 15 μM in cells maintained in primary culture [1] and organotypic culture [31], respectively.

The reasons for the differences in disaturated PC synthesis in the different type II cell preparations are not known. However, there are some interesting possibilities. It is possible that, due to the relatively long period of incubation, type II cells maintained in cell culture become more depleted of disaturated PC than are cells which are freshly isolated. If this is the case, the rate of disaturated PC synthesis may be greatly increased in cultured cells and these cells may exhibit a more linear time course, a greater magnitude, and a different palmitate dependence of incorporation. In this regard, we find that our cells contain 1.25 ($\pm$0.07) μg phosphorus (P) as phospholipid per 10^6 cells and 0.35 ($\pm$0.03) μg P as disaturated PC per 10^6 cells. These values are almost identical to those obtained by Mason et al. [24] in cells freshly isolated via density gradients (no cell culture was employed). Unfortunately, data for cells isolated by cell culture techniques are not yet available. Another reason for the differences between type II cell preparations may be that the techniques used for isolation could result in the selection of different populations of cells. For example, during primary cell culture, cells are obtained based on their ability to adhere to the culture dish, while the centrifugal elutriation process distinguishes cells on the basis of size. In addition, another possible reason for differences is the fact that our cells are in suspension while cultured cells adhere to the culture dish during the period of synthesis.

The results of our experiments also provide some information about factors which may be involved in the regulation of surfactant synthesis. In the presence of palmitate, there are three major substrates utilized in the synthesis of disaturated PC, viz., glucose, palmitate and choline [9]. We studied the effects of varying the concentrations of palmitate and choline on the incorporation of palmitate into disaturated PC while holding glucose at the normal plasma level of 5 mM. The relationship between extracellular palmitate and incorporation displays saturation kinetics with a $K_{1/2}$ of 0.10 mM and a V_{max} of 1.66 nmol/4.2 $\cdot$ 10^5 cells per 4 h. The normal plasma level of free fatty acids in the rat is 1.5 g/l [33]. If one assumes that 50% of the fatty acids is palmitate, then the plasma level of palmitate is about 3 mM. Thus, in vivo type II cells should be working in the range of the V_{max}. In the presence of choline, incorporation of palmitate into disaturated PC is enhanced at all levels of extracellular palmitate. The maximal effect of choline occurs at 0.05 mM, a value which is close to the normal plasma level of 0.025 mM in the rat [34]. Therefore, these data demonstrate that disaturated PC synthesis is dependent upon the the substrate levels and that the substrate levels which produce maximal rates of synthesis are close to the concentrations normally found in plasma.

The presence of the divalent cations, calcium and magnesium, also seems to influence incorporation of palmitate into disaturated PC. In the presence of Ca^{2+} alone, there is inhibition of incorporation. On the other hand, addition of Mg^{2+} reverses the inhibitory effects of Ca^{2+} and addition of Mg^{2+} alone seems to stimulate incorporation slightly. Although the reasons for these effects are not known, there are some possible explanations. It has been shown that Ca^{2+} inhibits the activity of cholinephosphotransferase, the enzyme responsible for the conversion of CDPcholine to phosphatidylcholine, in rat lung microsomes [35] and in isolated alveolar type II cells from rat [36]. In addition, Zachman [37] has demonstrated that Ca^{2+} inhibits and Mg^{2+} stimulates the activity of cholinephosphotransferase in human neonatal lung tissue. Therefore, the effects of Ca^{2+} and Mg^{2+} on incorporation of palmitate into disaturated PC in isolated alveolar type II cells may be due to their effects on enzyme activity.

The results of our experiments suggest that there is no direct effect of β-adrenergic or cholinergic agonists on surfactant synthesis in type II cells. It is possible that no effect was observed because the membrane receptors were destroyed during enzymatic digestion of the lungs. However, several points argue against this: (1) elastase spares β-adrenergic receptors in the hands of other investigators [4]; (2) atropine blocks the acetylcholine effect in our experiments; (3) cyclic AMP, which has been shown to increase during β-adrenergic stimulation of type II cells [5], has no effect; and (4) prolactin, a hormone which exerts its effect after binding to cell membranes, stimulates disaturated PC synthesis in our type II cell preparations (data not shown). Mettler et al. [10] reported β-adrenergic but not cholinergic stimulation of phosphatidylcholine synthesis in type II cells isolated

by primary cell culture techniques. Their results differ from ours in that we observed no β-adrenergic stimulation. The reason for this discrepancy is not known although the differences in the type II cell preparations may be important.

One factor which may be very important in the regulation of surfactant synthesis in type II cells is the level of surface-active materials surrounding the cells. We have found that surfactant obtained from lavage fluid inhibits incorporation of palmitate and choline into disaturated PC. At the present time the component(s) of surfactant responsible for the inhibition is not known. However, it does not seem to be disaturated PC itself. Some, but not all, of the inhibition can be accounted for when some of the lipids which are present in lavage fluid are added to the incubation medium in vesicular form. It is possible that some other substance which is present in surface-active materials is partially responsible for the inhibition, e.g., proteins. It is also possible that the form of the lipids and proteins, e.g., tubular myelin, is important in order for inhibition to occur. Nevertheless, there does seem to be some negative feedback in the control of surfactant synthesis in type II cells, possibly due to uptake of surfactant components and down regulation of de novo synthesis.

Some of the disaturated PC which has been synthesized during a 2 h period is broken down inside type II cells over the next 3 h if the cells are suspended in palmitate-free medium. The catabolism of disaturated PC can be prevented by slowing cellular metabolism at lower temperatures. Young et al. [38] reported that in vivo virtually all intracellular disaturated PC which is found in lamellar bodies is ultimately secreted onto the alveolar surface. Therefore, the disaturated PC which is metabolized in our experiments has probably not yet been packaged into lamellar bodies. In fact, the nonlinear time courses for incorporation of palmitate into disaturated PC (Fig. 1) may reflect a combination of synthesis and catabolism. The catabolism of disaturated PC is greatly slowed when palmitate is included in the incubation medium. One possible explanation for this is that an equilibrium, i.e., palmitate $\leftrightarrows$ palmitate in disaturated PC, exists if the disaturated PC has not been packaged into lamellar bodies. Thus, by removing palmitate the equilibrium is shifted to the left and vice versa. The products which are formed from the destruction of disaturated PC are not yet known. However, it is possible that an in vitro system such as this could be used to study the mechanisms involved in the catabolism of surfactant by alveolar type II cells. These reactions may be involved in the recycling of surfactant materials [39,40].

Our type II cell preparations are contaminated with alveolar macrophages (5%). It has been reported previously by other investigators [41,42] and by us [43] that incorporation of palmitate into disaturated PC occurs in alveolar macrophages. Therefore, it is possible that this contamination can account for some of the synthesis which apparently occurs in type II cells. However, we have calculated that this contamination could account for less than 4% of disaturated PC synthesis in our type II cell preparations. In addition, catabolism of disaturated PC occurs in alveolar macrophages [41]. Thus, it is also possible that the breakdown of disaturated PC which apparently occurs in type II cells (Fig. 4) is due to alveolar macrophage contamination. Two points argue against this: (1) the catabolism seems to occur in the intracellular compartment of type II cells and (2) addition of more alveolar macrophages to the type II cell preparation prevents the breakdown of disaturated PC (data not shown). The latter effect may be due to incorporation of palmitate released from type II cells following catabolism of disaturated PC into disaturated PC in alveolar macrophages. Therefore, contamination with alveolar macrophages does not seem to be a significant factor in our experiments.

In summary, we have studied the synthesis of disaturated PC in alveolar type II cells isolated via centrifugal elutriation. There are some differences between our results and those obtained by other investigators who used cells maintained in primary cell culture. These differences occur in the time course, magnitude and palmitate dependence of [^{3}H]palmitate incorporation into disaturated PC. Our results also provide some new information with regard to factors which may be important in the regulation of surfactant synthesis. For example, disaturated PC synthesis is affected by substrate levels, enzyme levels and the external levels of Ca^{2+} and Mg^{2+}. It appears that cholinergic and

β-adrenergic agonists do not act directly on type II cells to increase synthesis. There also seems to be negative feedback, since the presence of surfactant materials inhibits synthesis. Also, there may be intracellular catabolism of disaturated PC which has been recently synthesized in type II cells. We are currently performing experiments to learn more about intracellular catabolism of disaturated PC.

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